Folate Metabolism in the Rat Liver during Regeneration after Partial Hepatectomy

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1. Folate metabolism was studied during the early phases of liver regeneration after partial hepatectomy in rats accustomed to eating during the first 8 h of a daily 12 h dark period. 2. The content of 5-CH₃-H₄folate was drastically decreased during the first hours of regeneration. 3. The total HCO-H₄folate coenzymes showed a constant decrease during the first 3 days of regeneration, and a continuous interconversion between 5-HCO-H₄folate and 10-HCO-H₄folate. 4. 10-HCO-H₄folate synthetase, serine hydroxymethyltransferase and 5,10-CH₂-H₄folate dehydrogenase activities were relatively low during the first hours after the operation, and increased only several hours later. 5. The increase in enzyme activities showed a stepwise pattern, apparently due to an interaction between the regeneration process and the controlled feeding schedules.

Previous studies on the regulation of folate metabolism have shown a clear effect of some hormones on the synthesis and availability of folate coenzymes (Pasquali *et al.*, 1970; Bovina *et al.*, 1971; Rovinetti *et al.*, 1972), giving some insights on the mode of action of these hormones at the level of synthesis of nucleic acids and proteins. Other vitamin-deficiency experiments have shown close metabolic relationships between folic acid and riboflavin (Bovina *et al.*, 1969), vitamin B₁₂ (Puddu & Marchetti, 1965) and biotin (Marchetti *et al.*, 1966) other than the nutritional ones (Marchetti, 1971).

Our interest has been directed towards the study of regulatory mechanisms of biosynthesis and utilization of folate coenzymes in rapidly growing tissues. They represent a more useful experimental situation, since intense biosynthetic processes occur in growing tissues. In the chick embryo, a continual increase in these coenzymes occurs during development, owing to an increased activity of the relevant biosynthetic pathways (Landi *et al.*, 1972).

In the present paper we report the results of experiments on the distribution of folate coenzymes and on the enzyme activities catalysing their synthesis during the early phases of liver regeneration after partial hepatectomy. Our experiments have been performed on rats maintained under controlled feeding conditions, in order to evaluate more precisely the quantitative phenomena caused by the regenerating condition. It was shown (Barbiroli *et al.*, 1974) that normal rats maintained under the controlled feeding schedules of Potter *et al.* (1968) also exhibit daily rhythms of the enzyme activities involved in folate metabolism.

Experimental

Materials

All chemicals, enzymes and coenzymes (except H₂folate and H₄folate*) were obtained commercially. H₂folate was prepared by reduction of the folate with dithionite (Futerman, 1957). H₄folate was prepared by catalytic hydrogenation of folate over platinum oxide in acetic acid (O'Dell *et al.*, 1947).

Animals

Male albino Wistar rats (7 weeks old), weighing 200–220 g, were used in the experiments. At 21 days of age they were housed, usually two per cage, in an air-conditioned, windowless room illuminated from 21:00 to 09:00 h. Food was supplied just before the lights were switched off, and was removed 8 h later according to the feeding schedule developed by Potter *et al.* (1968) and Potter (1970), and designated '8+16'. Water was supplied *ad libitum*.

Partial hepatectomies were performed under ether anaesthesia, with removal of the main lobes (68–70% of liver was excised) as described by Higgins & Anderson (1931). The operations were performed at 21:00 h ± 30 min.

At intervals ranging between 6 and 72 h after partial hepatectomies, the rats were killed by decapitation,

* Abbreviations: H₂folate, dihydrofolate; H₄folate, tetrahydrofolate; 5,10-CH₂-H₄folate, 5,10-methylene-tetrahydrofolate; 10-HCO-H₄folate, 10-formyltetrahydrofolate; 5,10-CH=H₄folate, N⁵N¹⁰-methylidynetetrahydrofolate; 5-CH₃-H₄folate, 5-methyltetrahydrofolate.

and the livers were quickly removed, promptly frozen, and stored at -80°C for a maximum of 3 days. No difference in any enzyme activity studied nor in coenzyme pools was found in livers stored for over 1 week under these conditions.

Determination of folate coenzymes

To determine the folate coenzymes, liver acetone-dried powders were extracted with 1% (w/v) potassium ascorbate, pH 6.0, at 75°C for 30 min. The extracts were applied on a DEAE-cellulose-Hyflo Supercell (BDH Chemicals, Poole, Dorset, U.K.) (1:1, w/w) column ($20\text{cm} \times 1\text{cm}$) and eluted with ascorbate-phosphate buffer (0.2% potassium ascorbate, pH 6.0, and 0.5M-potassium phosphate buffer, pH 6.0). The eluted fractions were assayed for their ability to support the growth of the three test organisms, namely *Pediococcus cerevisiae* A.T.C.C. 8081, *Streptococcus faecalis* A.T.C.C. 8043 and *Lactobacillus casei* A.T.C.C. 7469 as previously described (Marchetti *et al.*, 1965).

Enzyme assays

For assaying H_4 folate dehydrogenase (EC 1.5.1.3), the tissues were homogenized in 4 vol. of 0.01M-Tris-HCl buffer, pH 7.0, and centrifuged at $20000g_{\text{av}}$ for 10 min at 4°C . The enzyme activity was determined in the supernatant by measuring the decrease in E_{340} caused by the conversion of NADPH into NADP^+ and of H_2 folate into H_4 folate (Mathews *et al.*, 1963).

For the assay of other enzyme activities, the tissues were homogenized in 9 vol. of 0.05M-Tris-HCl buffer, pH 7.5, and centrifuged at $10000g_{\text{av}}$ for 30 min at 4°C . Serine hydroxymethyltransferase (EC 2.1.2.1) activity was assayed in the supernatant by measuring colorimetrically both the free and the bound formaldehyde in 5,10- CH_2 - H_4 folate with the acetylacetone reagent (Scrimgeour & Huennekens, 1962). 5,10- CH_2 - H_4 folate dehydrogenase (EC 1.5.1.5) activity was assayed by determining spectrophotometrically at 355 nm the 5,10- $\text{CH}=\text{H}_4$ folate formed in the system (Scrimgeour & Huennekens, 1963). 10-HCO- H_4 folate synthetase (EC 6.3.4.3)

activity was assayed in the supernatant after partial purification with protamine sulphate and solid $(\text{NH}_4)_2\text{SO}_4$, by measuring the 5,10- $\text{CH}=\text{H}_4$ folate formed in the reaction mixture (Rabinowitz & Pricer, 1963).

Protein was determined by the colorimetric method of Lowry *et al.* (1951), with crystalline bovine plasma albumin as the standard.

Results

Folate coenzymes

Table 1 shows the total and reduced folate forms in the rat liver during the first 3 days of regeneration after partial hepatectomy. An initial decrease in the total and reduced folate forms was found 12 h after partial hepatectomy. This was followed by a normal value between 24 and 48 h, which decreased steadily in the next period studied (60–72 h). As far as the individual folate forms are concerned, the H_4 folate (Fig. 1a) did not show any remarkable variation in the times studied during regeneration, whereas the 5- CH_3 - H_4 folate (Fig. 1a) decreased drastically in the first 12 h after the operation and reached the normal value again 12 h later.

Fig. 1(b) shows the behaviour of the HCO- H_4 folate forms. The total HCO- H_4 folate showed an almost constant decrease from the normal value until the third day of regeneration, whereas the individual forms (5-HCO- H_4 folate and 10-HCO- H_4 folate) behaved in opposite ways. During the first 24 h of regeneration, both exhibited a parallel decrease. At 36 h, the 10-HCO- H_4 folate was still following this pattern, whereas the 5-HCO- H_4 folate increased over and above the normal value. This opposite behaviour was also found during the next 36 h of regeneration.

Enzyme activities

Fig. 2 shows the behaviour of enzyme activities of folate metabolism during the early phases of liver regeneration.

H_4 folate dehydrogenase activity (EC 1.5.1.3) studied during the first 36 h of regeneration did not

Table 1. Folate coenzyme content of rat liver during regeneration after partial hepatectomy

For the methods of determination of folate coenzymes see the Experimental section. Data represent the means of three determinations on pooled livers (three or four livers per pool) and are expressed as $\mu\text{g/g}$ wet wt. $\pm$ S.E.M.

Folate forms	Time after operation (h) ...	Content ($\mu\text{g/g}$ wet wt.)						
		0	12	24	36	48	60	72
Total folate		6.37 $\pm$ 0.53	5.33 $\pm$ 0.51	6.33 $\pm$ 0.57	6.75 $\pm$ 0.61	6.65 $\pm$ 0.53	5.83 $\pm$ 0.49	5.27 $\pm$ 0.41
Reduced forms		5.75 $\pm$ 0.51	4.80 $\pm$ 0.40	5.72 $\pm$ 0.60	6.10 $\pm$ 0.50	5.09 $\pm$ 0.41	5.26 $\pm$ 0.45	4.74 $\pm$ 0.39
Non-reduced forms		0.62 $\pm$ 0.06	0.53 $\pm$ 0.06	0.61 $\pm$ 0.05	0.65 $\pm$ 0.05	0.56 $\pm$ 0.04	0.57 $\pm$ 0.05	0.52 $\pm$ 0.05

Fig. 1. Content of folate derivatives in livers of rats adapted to the '8+16' feeding schedules and subjected to 70% partial hepatectomy at 21:00h

Each point represents the mean of three determinations on pooled livers (three to four livers per pool) (S.E.M. values are indicated by vertical bars). At zero time the excised livers were used. In Figs. 1 and 2, cross-hatched bars represent the dark period (from 09:00h to 21:00h actual time) in 24h cycles and the open bars below them represent the period when food was available. (a) ●, H_4 folate; ○, $5-CH_3-H_4$ folate; (b) ■, total $HCO-H_4$ folate forms; □, $10-HCO-H_4$ folate; △, $5-HCO-H_4$ folate.

show any large variation over the daily pattern of control rats (Fig. 2a). Serine hydroxymethyltransferase activity (EC 2.1.2.1) showed a general increase during the first 48h after the operation (Fig. 2b). This increase was not constant and showed successive peaks of activity at the time of day in which normal rats trained to the '8+16' feeding schedule of Potter (1970) exhibited the daily diurnal variation.

The same kind of pattern was also observed in the $5,10-CH_2-H_4$ folate dehydrogenase activity (EC 1.5.1.5) (Fig. 2c). A first peak of activity was found 6h after the operation, after which time the increase in this enzyme activity followed a stepwise pattern during the next two post-operative days.

A somewhat different behaviour was found for the $10-HCO-H_4$ folate synthetase activity (EC 6.3.4.3) (Fig. 2d). A background activity was maintained throughout the first post-operative day, and a step-

Fig. 2. Activities of enzymes of folate metabolism in livers of rats adapted to the '8+16' feeding schedule and subjected to 70% partial hepatectomy at 21:00h

Each point represents the mean for six rats; S.E.M. values are indicated by vertical bars. The excised livers were used to determine the activities at zero time. Dashed lines show the daily pattern of the enzyme activities found in intact rats (Barbiroli *et al.*, 1974). For experimental details, see the legend to Fig. 1 and the Experimental section. (a) H_4 folate dehydrogenase activity; (b) serine hydroxymethyltransferase activity; (c) $5,10-CH_2-H_4$ folate dehydrogenase activity; (d) $10-HCO-H_4$ folate synthetase activity.

wise increase was found during the following 2 days of regeneration.

Sham-operated rats did not show any variation of the enzyme activities studied when compared with intact rats.

Discussion

Folate metabolism appears to be modified remarkably during the early phases of liver

regeneration after partial hepatectomy, with respect to either folate coenzyme concentrations or enzyme activities connected with folate metabolism.

The content of 5-CH₃-H₄folate was drastically decreased during the first hours of regeneration. This was matched during the same time-period by a low 10-HCO-H₄folate synthetase activity. Also serine hydroxymethyltransferase and 5,10-CH₂-H₄folate dehydrogenase, which catalyse a subsidiary biosynthetic pathway of folate coenzymes, showed a relatively low activity. The remarkable decrease in 5-CH₃-H₄folate immediately after the operation can be attributed to a sudden large requirement for the active forms of folate. The methylated form can be regarded as a sort of reservoir from which the more active forms are obtained. Therefore the recovery to the normal concentration of folate coenzymes, which occurred during the succeeding hours, indicates a more direct synthesis of the active forms by the specific enzyme activities induced by the regeneration process. This possibility is also supported by the behaviour of the enzyme activities studied. In general they did not show any large increase during the first period of regeneration, whereas their subsequent increased activity corresponded to the normalization of the 5-CH₃-H₄folate content.

The enzyme activities also showed a pattern which is not linear during the time-period studied, but which was characterized by successive peaks. This behaviour is apparently due to the interaction between the regeneration process and the daily rhythm of these enzyme activities found in the normal liver (Barbiroli *et al.*, 1974), and which is induced by the signals of the controlled feeding schedules.

Serine hydroxymethyltransferase and 5,10-CH₂-H₄folate dehydrogenase activities showed, after the first post-operative day, a pattern resulting from the overlapping of the increases caused by the regeneration process with those induced by the controlled-feeding schedules, whereas 10-HCO-H₄folate synthetase activity showed this interaction only from day 2 onwards.

Our present findings show that, during the early phases of liver regeneration, the methylfolate pool is large enough to meet the sudden large requirement for folate coenzymes needed for biosynthesis of macromolecules. Besides this, while the coenzyme pools are in general decreasing, the enzyme activities leading to their biosynthesis are increasing, indicating a

progressive and large utilization of the coenzymes. Further, in view also of the interaction between food intake and regulation of nucleic acid metabolism either in normal (Barbiroli *et al.*, 1975) or regenerating rat liver (Hopkins *et al.*, 1973), these results further point out the need for a precise control of the feeding schedules on which the animals are maintained in all experiments dealing with liver metabolism.

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